



$G\alpha_q$ and $G\alpha_{11}$ proteins mediate endothelin-1 signaling in neural crest-derived pharyngeal arch mesenchyme

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Abstract

Endothelin-A (ET_A) is a G-protein-coupled receptor expressed in the neural crest-derived mesenchyme of the pharyngeal arches during craniofacial development. Targeted deletion of the ET_A receptor or its ligand endothelin-1 (ET-1) causes cleft palate and hypoplasia of the mandible, otic cup, and tympanic ring. Previously we showed that $G\alpha_q/G\alpha_{11}$ -null mice die around E11.0, whereas $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$ mice survive to birth with hypomorphic phenotypes similar to, but less severe than, ET_A or $ET-1$ -null mice. To determine whether ET-1 signaling is transduced by $G\alpha_q/G\alpha_{11}$ proteins, we examined the expression patterns of several ET-1 dependent and independent transcription factors in $G\alpha_q/G\alpha_{11}$ -deficient embryos. Expression of genes encoding the ET-1-dependent transcription factors *Dlx3*, *Dlx6*, *dHAND*, and *eHAND* was specifically downregulated in the pharyngeal arches of $G\alpha_q/G\alpha_{11}$ -deficient mice. In contrast, pharyngeal arch expression of the homeobox gene *Msx1*, which is not regulated by ET-1 signaling, was maintained in these embryos. We conclude that the $G\alpha_q$ and $G\alpha_{11}$ proteins serve as the intracellular mediators of ET-1 signaling in the pharyngeal arch mesenchyme.

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Introduction

Endothelin-A (ET_A) is a G-protein-coupled receptor expressed in the neural crest-derived mesenchyme of the pharyngeal arches during development. The ligand for ET_A , endothelin-1 (ET-1), is expressed in pharyngeal arch epithelium and binds the receptor after cleavage from its pro-form by the membrane-bound metalloprotease, endothelin converting enzyme-1 (ECE-1) (Xu et al., 1994). Activation of the ET_A receptor by ET-1 is required for proper development of neural crest-derived craniofacial structures, the cardiac outflow tract, and the great vessels. Proliferation and differentiation of cell populations contributing to these

structures are determined by expression of transcription factors including gooseoid, *Dlx2*, *Dlx3*, *dHAND*, *eHAND*, and *Barx1*, all of which require ET-1 signaling for correct spatiotemporal expression in the pharyngeal arches.

Various components of the endothelin signaling pathway have been inactivated in vivo allowing study of their roles in development of neural crest cells. Mice with deficiencies in the genes encoding ET-1 (*Edn-1*) (Kurihara et al., 1994, 1995), ET_A (Clouthier et al., 1998), or ECE-1 (Yanagisawa et al., 1998) die shortly after birth with abnormal craniofacial bones, cleft palate, malformed thyroid and thymus, aortic arch anomalies, and ventricular septal defects. These morphologic abnormalities correlate with the restricted expression patterns of ET-1 and ET_A . Signals from the ET_A receptor can potentially be transmitted by broadly expressed G-proteins of the Gq, Gi, and/or Gs class; however, little is known regarding the mechanisms of selectivity in ET_A -G-

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protein coupling. Studies of ET-1 signaling in the context of myocardial hypertrophy have shown that ET-1 interaction with ET_A stimulates phospholipase C- β (PLC- β) isoforms through activation of the G_q class of G-proteins. Indeed, adult cardiomyocyte hypertrophy is suppressed in mice with a cardiomyocyte-specific deletion of both $G\alpha_q$ and $G\alpha_{11}$ (Wettschureck et al., 2001). By contrast, $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ mice die around E11.0 apparently due to cardiomyocyte hypoplasia and heart failure. Interestingly, craniofacial development initiates prior to death, allowing us to assess the role of $G\alpha_q/G\alpha_{11}$ on the ET-1 signaling pathway in the pharyngeal arch mesenchyme.

We showed previously that combined inactivation of $G\alpha_q$ and $G\alpha_{11}$ in mice results in alterations of neural crest-derived structures similar to, but less severe than, those observed upon disruption of ET-1 signaling (Offermanns et al., 1998). Mice with a single active allele of $G\alpha_q$ or $G\alpha_{11}$ survive up to 2 h after birth but are born small, anoxic, and unresponsive to tactile stimuli. In addition, $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$ neonates exhibit malformation of neural crest derived craniofacial structures, including the mandible, tympanic ring, and bones of the otic cup. These craniofacial malformations are not as severe as those caused by the absence of ET-1 or ET_A, presumably because one copy of $G\alpha_{11}$ transduces an insufficient fraction of the normal ET-1 signal. Normal pharyngeal development requires either a single copy of $G\alpha_q$ or two copies of $G\alpha_{11}$. Both $G\alpha_q$ and $G\alpha_{11}$ are widely expressed and appear to have overlapping functions (Offermanns et al., 1998; Wilkie et al., 1991). Despite reduced fecundity, ataxia, and platelet aggregation disorders in $G\alpha_q^{(-/-)}$ mice (Offermanns et al., 1997a, 1997b), any combination of two functional alleles of $G\alpha_q$ or $G\alpha_{11}$ is sufficient for survival, normal craniofacial development, and production of fertile adults.

The similarity of developmental defects seen in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$ mice compared to *Edn-1* or ET_A-null mice led us to hypothesize that the $G\alpha_q$ and $G\alpha_{11}$ proteins are the primary intracellular transducers of ET-1 signaling in the cranial neural crest. Here we show that expression of ET_A-dependent genes is altered in mice harboring inactive alleles of $G\alpha_q$ and $G\alpha_{11}$, while expression of other transcription factors in the pharyngeal arches remains unchanged, indicating that the $G\alpha_q$ and $G\alpha_{11}$ proteins mediate ET-1 signaling in the pharyngeal arches.

Materials and methods

Harvesting and genotyping of embryos

$G\alpha_q^{(+/-)}G\alpha_{11}^{(+/-)}$ mice were intercrossed and embryos were collected at E9.5. Embryos were fixed in 4% paraformaldehyde overnight and stored at -20°C . Yolk sac DNA was genotyped for $G\alpha_q$ and $G\alpha_{11}$ by polymerase chain reaction (PCR). $G\alpha_q$ genotyping was performed using two

forward primers, [TW140: 5'-AGGGCCCATGAGGACATGTATGC-3'], specific for the wild-type allele, and [TW36: 5'-AGGATCTCGTCGTGACCCATGGCGA-3'], specific for the knockout allele. The reverse primer, [TW71: 5'-TTCAAAGTATCACACTCACATCACAG-3'], is common to both alleles of $G\alpha_q$. $G\alpha_{11}$ genotyping was similarly achieved using two forward primers, [TW141: 5'-GACACTGCCATCTGTACAAGG-3'] and [α PGK: 5'-GCTAAGCGCATGCTCCAGAC-3'], in combination with the reverse primer [CT26: 5'-GAGAATGACAGACGAGTTCTG-3']. Touchdown PCR was carried out under the following conditions: 94°C for 4 min; 94°C for 30 s; 72°C for 30 s; and 72°C for 30 s (10 cycles, decreasing the annealing temperature by 2°C each cycle after the first cycle); 94°C for 30 s; 55°C for 30 s; and 72°C for 30 s (20 cycles) and 72°C for 7 min. $G\alpha_q$ wild-type and null alleles yielded products of 700 and 400 bp, respectively, while $G\alpha_{11}$ wild-type and null alleles yielded products of 410 and 300 bp, respectively.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Yamagishi et al., 1999) using digoxigenin-labeled antisense riboprobes synthesized from *Dlx3*, *dHAND*, and *eHAND* cDNAs linearized with *NotI* and transcribed with T3 polymerase. The *Msx1* riboprobe was generated from cDNA linearized with *BamHI* and transcribed with T3 polymerase.

Radioactive section in situ hybridization

^{35}S -labeled antisense riboprobes were synthesized from partial cDNAs of *Msx1*, *Msx2*, *Dlx3*, *Dlx6*, *dHAND*, and *eHAND*. cDNAs were linearized and transcribed using the following restriction enzymes and RNA polymerases: *Msx1-BamHI*, T7; *Msx2-EcoRV*, SP6; *Dlx3-NotI*, T3; *Dlx6-SpeI*, T7; *dHAND-EcoRI*, SP6; *eHAND-BamHI*, T7 (MAXIScript; Ambion Inc.). Radioactive section in situ hybridization was performed on paraffin-embedded sections of E9.5 mouse embryos using these riboprobes, as previously described (Nakagawa et al., 1999).

Results

HAND gene expression is downregulated in pharyngeal arches of $G\alpha_q/G\alpha_{11}$ -deficient embryos

Expression of the basic helix-loop-helix transcription factors *dHAND* and *eHAND* is downregulated in *Edn1*-null or ET_A-null embryos (Clothier et al., 2000; Thomas et al., 1998), leading us to examine the effect of $G\alpha_q/G\alpha_{11}$ deficiency on expression of these two genes. Whole-mount in situ hybridization revealed expression of both *HAND* genes in wild-type, $G\alpha_q^{(+/-)}G\alpha_{11}^{(+/-)}$ and $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$

(Fig. 1). *dHAND* was expressed broadly in the pharyngeal arches and was robust in all mutants containing a single allele of either $G\alpha_q$ or $G\alpha_{11}$. The expression of *eHAND* was restricted to the very medial portion of the pharyngeal arch of mutants and wild-type and was slightly diminished in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$, although clearly detectable. In contrast, neither *dHAND* nor *eHAND* gene expression was detectable in $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ embryos by whole-mount in situ hybridization, even in frontal view (Fig. 1).

To examine the regulation of *HAND* gene expression in the pharyngeal arch mesenchyme, we performed radioactive section in situ analysis on serial sections from various $G\alpha_q/G\alpha_{11}$ mutant embryos. High levels of *dHAND* expression were observed in the wild-type, $G\alpha_q^{(+/ -)}G\alpha_{11}^{(+/ -)}$, and $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$ sections, whereas *dHAND* expression was not detectable in the $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ sections, consistent with the whole-mount studies. *eHAND* expression was similarly maintained in wild-type and $G\alpha_q^{(+/ -)}G\alpha_{11}^{(+/ -)}$ embryos, but was not detected in the $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$ or $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ sections examined. Given the clear expression of *eHAND* in the medial portion of $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$ pharyngeal arches by whole-mount in situ hybridization, the absence of *eHAND* expression in the $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$ section most likely reflects a failure to capture the narrow medial domain of *eHAND* expression in coronal sections. Nevertheless, combined data from the whole-mount and section in situ experiments suggest that *eHAND* is downregulated in the pharyngeal arches of mice lacking both alleles of $G\alpha_q$ or $G\alpha_{11}$ but that either allele is sufficient to direct some *HAND* gene expression.

Msx1 expression is maintained in the pharyngeal arches of $G\alpha_q/G\alpha_{11}$ -deficient embryos

Pharyngeal arch expression levels and patterns of other genes have been evaluated in *Edn1*-null mice. We examined expression of several of these genes on whole-mount or serial sections from the same embryos used for *HAND* gene expression analysis. As previously described for *Edn1*-null embryos, expression of the homeobox gene *Msx1* was maintained in $G\alpha_q/G\alpha_{11}$ -deficient embryos as observed by whole-mount in situ hybridization (Fig. 2). Maintenance of *Msx1* expression in $G\alpha_q/G\alpha_{11}$ -deficient embryos confirms that there is not an overall differentiation defect or a deficiency of cells in the pharyngeal arches of $G\alpha_q/G\alpha_{11}$ mutants and implies that the reduction of *HAND* gene expression observed in these embryos specifically reflects the absence of $G\alpha_q/G\alpha_{11}$ signaling.

Dlx3 and *Dlx6* expression is downregulated in $G\alpha_q/G\alpha_{11}$ -deficient embryos and maintained in *dHAND*-null embryos

Expression of *Dlx3* and *Dlx6*, two homeobox-containing genes expressed in the distal mesenchyme and epithelium of the pharyngeal arches, is absent in the first and second

arches of E9.5 *ET_A*-null embryos (Charité et al., 2001; Clouthier et al., 2000). *Dlx6* binds an *ET_A*-dependent neural crest enhancer required for expression of *dHAND* in the pharyngeal arches (Charité et al., 2001). To determine whether *Dlx3* and *Dlx6* are also downregulated in $G\alpha_q/G\alpha_{11}$ mutant embryos, we examined their expression in E9.5 embryos with deletions of $G\alpha_q$ and/or $G\alpha_{11}$ genes. Whole-mount in situ hybridization revealed expression of *Dlx3* in wild-type E9.5 embryos, reduced expression levels in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$ embryos, and complete lack of expression in $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ embryos (data not shown).

We also examined *Dlx3* and *Dlx6* expression by radioactive section in situ hybridization on the various $G\alpha_q/G\alpha_{11}$ mutant embryos (Fig. 3). *Dlx3* expression was strong in wild-type sections and slightly diminished in sections from $G\alpha_q^{(+/ -)}G\alpha_{11}^{(+/ -)}$ embryos. Inactivation of three $G\alpha_q$ and $G\alpha_{11}$ alleles led to a further decrease in *Dlx3* expression consistent with the craniofacial defects of $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$ mice. *Dlx3* expression was not detected in sections from $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ embryos. In contrast, *Dlx6* expression was normal in double heterozygous embryos, barely detectable in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$ and undetectable in double-null pharyngeal arches. These findings are consistent with the increasing severity of phenotype observed as alleles of both $G\alpha_q$ or $G\alpha_{11}$ are deleted and, together with the data for the *HAND* genes and *Msx1*, provide evidence that $G\alpha_q$ and $G\alpha_{11}$ transduce *ET-1/ET_A* signaling in neural crest cells to promote development of the pharyngeal arches.

Expression of *dHAND* in the pharyngeal arches has previously been shown to be dependent on *ET-1* signaling (Thomas et al., 1998). To determine whether *dHAND* is also required for *Dlx3* expression in the pharyngeal arches, we examined the expression of *Dlx3* in E9.5 *dHAND*-null embryos. Pharyngeal arch expression of *Dlx3* was not diminished in *dHAND*-null embryos (Fig. 4B), compared to wild-type littermates (Fig. 4A). Likewise, *dHAND* expression was not diminished in *Dlx3*-null embryos (Fig. 4D). We conclude that $G\alpha_q/G\alpha_{11}$ mediates *ET-1* activation of *dHAND* and *Dlx3* in parallel pathways in neural crest cells of the pharyngeal arch during craniofacial development.

Discussion

The similarity of craniofacial structures that were affected in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/ -)}$, *ET_A*, and *ET-1*-null neonatal mice led us to investigate whether these genes may function in a common signaling pathway. We examined whether the pharyngeal arch expression of several transcription factors, which is disrupted when *ET-1* signaling is abolished, is similarly affected in $G\alpha_q/G\alpha_{11}$ -deficient embryos. We found this to be the case for all such transcription factors examined—*Dlx3*, *Dlx6*, *dHAND*, and *eHAND*. Interestingly, while pharyngeal arch expression of these homeodomain and bHLH genes is diminished in $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$, *ET_A*, and *ET-1*-null embryos, it is maintained in other de-

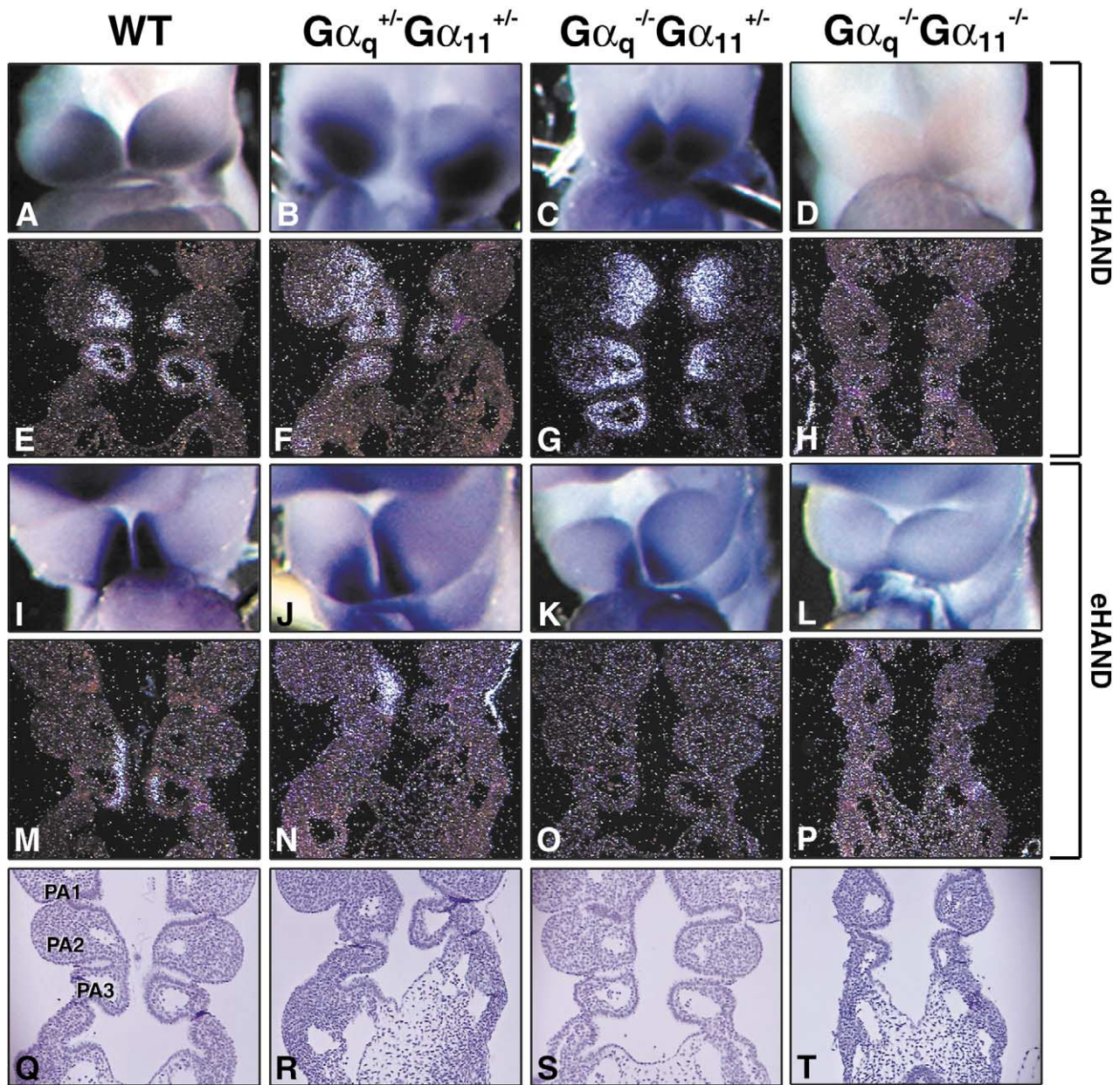


Fig. 1. *HAND* genes are downregulated in $G\alpha_q/G\alpha_{11}$ -deficient pharyngeal arches. Whole-mount in situ hybridization of *dHAND* on pharyngeal arches of wild-type (A), $G\alpha_q^{+/-}G\alpha_{11}^{+/-}$ (B), $G\alpha_q^{-/-}G\alpha_{11}^{+/-}$ (C), or $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ (D) E9.5 mouse embryos. Radioactive in situ hybridization of *dHAND* on coronal sections of wild-type (E), $G\alpha_q^{+/-}G\alpha_{11}^{+/-}$ (F), $G\alpha_q^{-/-}G\alpha_{11}^{+/-}$ (G), or $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ (H) E9.5 mouse embryos through the pharyngeal arches. Whole-mount in situ hybridization of *eHAND* on pharyngeal arches of wild-type (I), $G\alpha_q^{+/-}G\alpha_{11}^{+/-}$ (J), $G\alpha_q^{-/-}G\alpha_{11}^{+/-}$ (K), or $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ (L) E9.5 mouse embryos. Radioactive in situ hybridization of *eHAND* on coronal sections of wild-type (M), $G\alpha_q^{+/-}G\alpha_{11}^{+/-}$ (N), $G\alpha_q^{-/-}G\alpha_{11}^{+/-}$ (O), or $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ (P) E9.5 mouse embryos. Corresponding bright field images are shown (Q–T). Images in A–D and I–L are frontal views focusing on the pharyngeal arches. PA1, pharyngeal arch 1; PA2, pharyngeal arch 2; PA3, pharyngeal arch 3.

veloping structures including the limb buds and, in the case of *dHAND* and *eHAND*, the heart. The expression of important markers of the more posterior pharyngeal arches, such as *Dlx2* and *Barx1*, is similar in wild-type and $ET_A^{-/-}$ null mice until E10.5, making analysis in the $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ embryos impossible due to their early lethality. Given that $G\alpha_q/G\alpha_{11}$ is required for *HAND* gene expression during craniofacial development, we tested whether $G\alpha_q/$

$G\alpha_{11}$ signaling might also regulate the expression of bHLH genes required for neurogenesis (Guillemot, 1999) because we noted that mutant neonates expressing a single copy of either $G\alpha_q$ or $G\alpha_{11}$ exhibited phenotypes consistent with defective neural developmental (Offermanns et al., 1998). However, neural tube expression of the neurogenic bHLH transcription factors *Mash1*, *Math1*, *Ngn1*, and *Ngn2* appeared normal in $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ embryos at E9.5 (T.M.

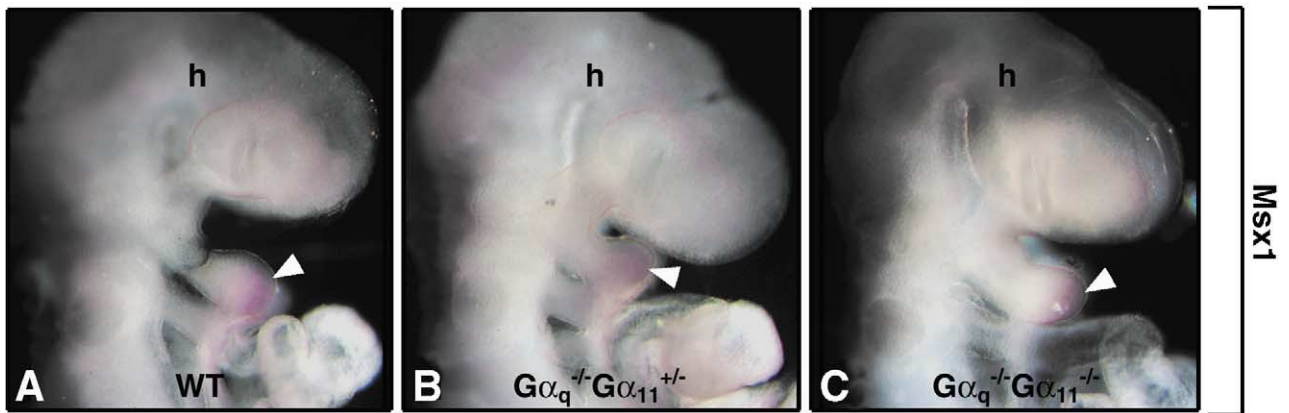


Fig. 2. Pharyngeal arch expression of *Msx1* in wild-type and $G\alpha_q/G\alpha_{11}$ -deficient mice. Whole-mount in situ hybridization of *Msx1* on wild-type (A), $G\alpha_q^{+/-}G\alpha_{11}^{+/-}$ (B), or $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ (C) E9.5 embryos. Arrowheads indicate the region of expression in the pharyngeal arch 1 (PA1); h, head.

Wilkie and J. Johnson, unpublished data). Thus, $G\alpha_q/G\alpha_{11}$ signaling was only found to be required for the expression of *Dlx* and *HAND* genes in the pharyngeal arches.

$G\alpha_q/G\alpha_{11}$ signaling regulates the activity of several transcription factors in various cell types, but little is known about the signaling hierarchy in cranial neural crest cells.

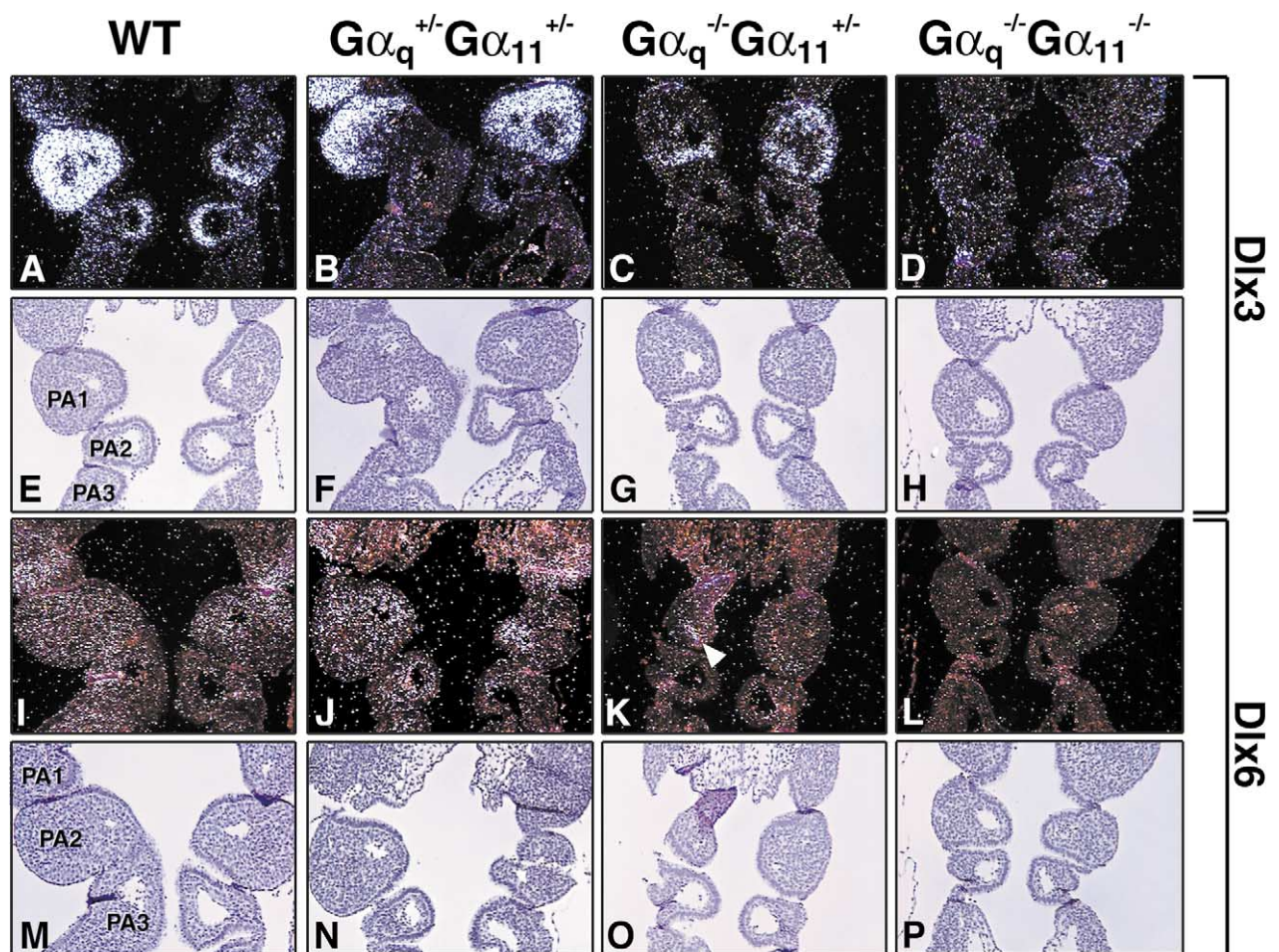


Fig. 3. *Dlx3* and *Dlx6* pharyngeal arch expression is regulated by $G\alpha_q/G\alpha_{11}$ in a dose-dependent manner. Radioactive in situ hybridization of *Dlx3* on coronal sections of wild-type (A), $G\alpha_q^{+/-}G\alpha_{11}^{+/-}$ (B), $G\alpha_q^{-/-}G\alpha_{11}^{+/-}$ (C), or $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ (D) E9.5 mouse embryos through the pharyngeal arches. Corresponding bright field images are shown (E–H). Radioactive in situ hybridization of *Dlx6* on coronal sections of wild-type (I), $G\alpha_q^{+/-}G\alpha_{11}^{+/-}$ (J), $G\alpha_q^{-/-}G\alpha_{11}^{+/-}$ (K), or $G\alpha_q^{-/-}G\alpha_{11}^{-/-}$ (L) E9.5 mouse embryos. Arrowhead indicates region of *Dlx6* expression captured on section. Corresponding bright field images are shown (M–P). PA1, pharyngeal arch 1; PA2, pharyngeal arch 2; PA3, pharyngeal arch 3.

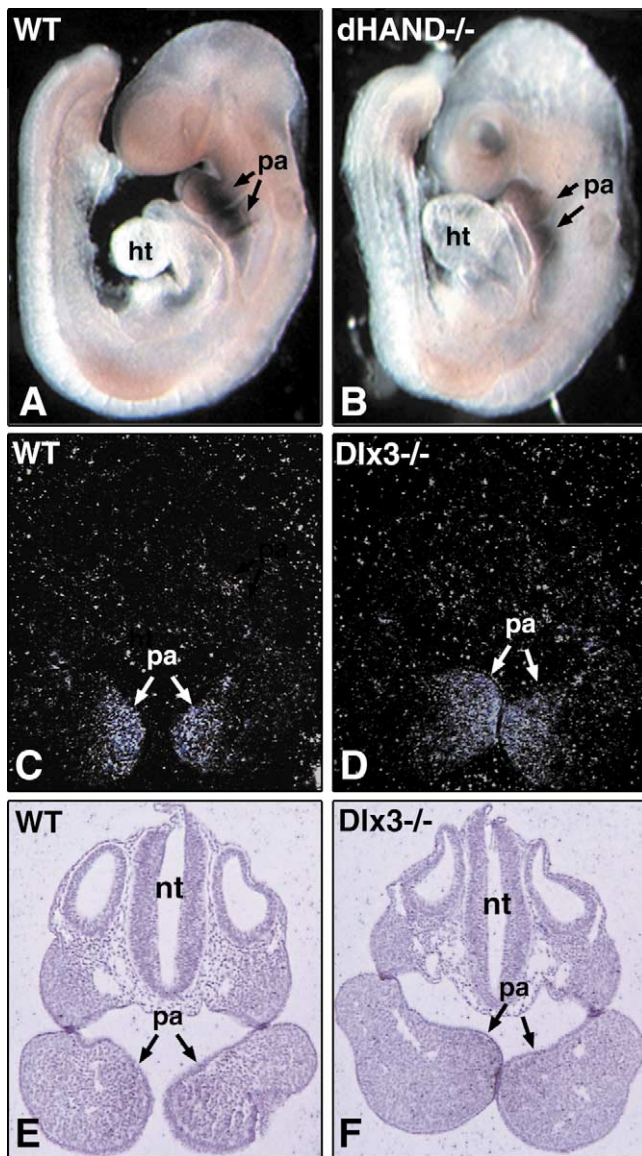


Fig. 4. *Dlx3* and *dHAND* function in independent pathways in developing pharyngeal arches. Whole-mount in situ hybridization of *Dlx3* on wild-type (A) or *dHAND*^(-/-) (B) E9.5 mouse embryos. Left lateral view is shown. Radioactive section in situ hybridization of *dHAND* on transverse sections of wild-type (C) or *Dlx3*^(-/-) (D) E9.5 embryos. Corresponding bright field images are shown (E and F). pa, pharyngeal arch; ht, heart; nt, neural tube.

$G\alpha_q$ and $G\alpha_{11}$ both activate PLC- β , which, in turn, catalyzes the hydrolysis of phosphatidylinositol biphosphate into diacylglycerol and inositol triphosphate (Srncka and Sternweis, 1993). This leads to intracellular calcium release and activation of protein kinase C (PKC), calmodulin-activated kinase II (CaMKII), and the Ca^{2+} -regulated phosphatase, calcineurin. These kinases and phosphatases modulate the activity of several proteins that regulate transcription. For example, $G\alpha_q/G\alpha_{11}$ regulates activation of the transcription factor Pit-1 in a Raf-1-dependent pathway to control expression of the prolactin gene (Tian et al., 1995). In lymphocytes and cardiomyocytes, NF-AT nuclear translocation and activation of gene transcription is stimulated by

calcineurin (Boss et al., 1996; Li et al., 1998; Molkenstein et al., 1998). In B cells, NF κ B-dependent transcription is stimulated by $G\alpha_q/G\alpha_{11}$ -mediated activation of the Ca^{2+} -sensitive tyrosine kinase Pyk2 (Shi and Kehrl, 2001). Additionally, in hypothalamic and retinal neurons, Gq-mediated depletion of phosphatidylinositol 4,5-bisphosphate (PIP₂) at the plasma membrane apparently regulates nuclear translocation of the DNA-binding protein, tubby (Santagata et al., 2001). The MEK2 transcription factor is also regulated by G protein, calcineurin, CaM kinase, and MAP kinase signaling (reviewed in McKinsey et al., 2002), making it an interesting potential target for $G\alpha_q/G\alpha_{11}$ signaling cascades in the developing neural crest. Analysis of PIP₂ and calcium-dependent genes, MAP kinase targets, and CaMKII and PKC substrates in the context of the pharyngeal arch may uncover additional transcription factors that regulate *Dlx3*, *Dlx6*, *dHAND*, and *eHAND* and provide a mechanism for their activation.

$G\alpha_q/G\alpha_{11}$ signaling in receptor complexes is dosage dependent in vivo. Inactivation of one or two of the four alleles of $G\alpha_q/G\alpha_{11}$, in any combination, results in a craniofacial morphology indistinguishable from wild-type. We found that transcription factors regulated by ET-1 maintain high expression levels in $G\alpha_q^{(+/-)}G\alpha_{11}^{(+/-)}$ embryos, reflecting the requirement of only two alleles of $G\alpha_q/G\alpha_{11}$ for correct development and survival. By contrast, a single active copy of $G\alpha_{11}$ (in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$ embryos) resulted in reduced expression of *Dlx3* and *Dlx6*, while complete $G\alpha_q/G\alpha_{11}$ -deficiency ablated expression of both *HAND* genes, in addition to *Dlx3* and *Dlx6*. This suggests a requirement of threshold levels of $G\alpha_q/G\alpha_{11}$ protein for functional ET-1 signaling to some targets. It is possible that $G\alpha_{11}$ is expressed at lower levels than $G\alpha_q$ in the neural crest derived mesenchyme of the pharyngeal arches, or couples ET_A receptor less efficiently, leading to the increased severity of defects observed in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$ compared to $G\alpha_q^{(+/-)}G\alpha_{11}^{(-/-)}$ embryos.

We also found that although $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$ neonates die shortly after birth with defects in neural crest derived structures, these embryos do express high levels of *dHAND*. This probably reflects a requirement for other proteins, in addition to *dHAND*, whose expression is regulated by $G\alpha_q/G\alpha_{11}$ signaling to support development of the pharyngeal arch mesenchyme. *Dlx3* is a candidate for this role as its expression is markedly reduced in $G\alpha_q^{(-/-)}G\alpha_{11}^{(+/-)}$ embryos and is excluded from the pharyngeal arches of $G\alpha_q/G\alpha_{11}$ -null embryos. *Dlx3* and *dHAND* may function in parallel $G\alpha_q/G\alpha_{11}$ -dependent pathways because *Dlx3* expression is normal in *dHAND*-null embryos and *dHAND* expression remains unaltered in *Dlx3*-null embryos (Figs. 4 and 5). It is likely that additional transcription factors regulated by ET-1 signaling are downregulated in $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ embryos and are required for development of neural crest derived structures.

A genomic region upstream of *dHAND* containing four homeodomain binding sites is necessary and sufficient for

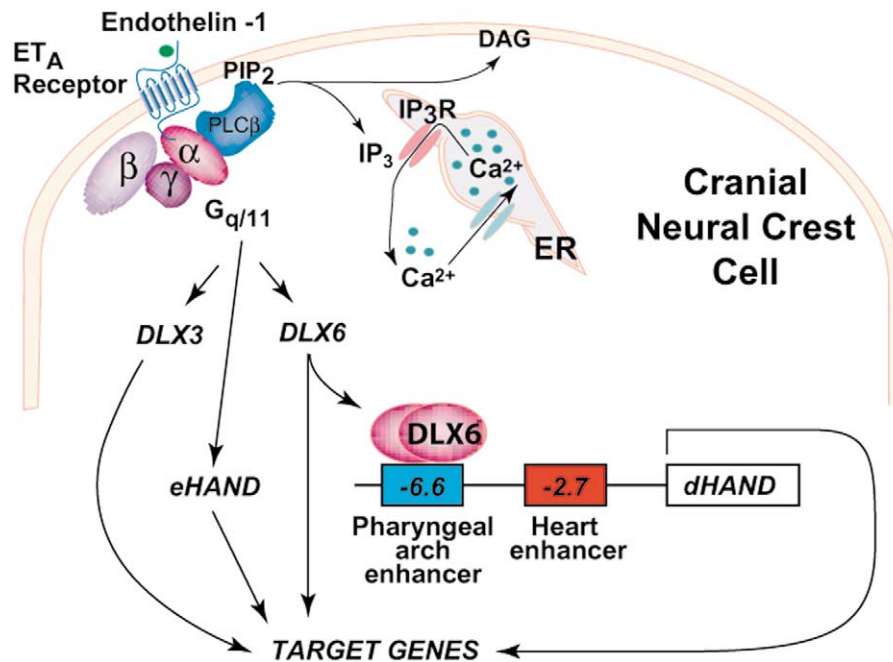


Fig. 5. Model of $G\alpha_q/G\alpha_{11}$ -coupled ET_A activation of *Dlx* and *HAND* expression in pharyngeal arch mesenchyme. Endothelin-1 is expressed in the pharyngeal endoderm and binds the endothelin A (ET_A) receptor in neural crest cells of the pharyngeal arches of E9.5 embryos (Clouthier et al., 1998). Endothelin signaling in neural crest cells is coupled by heterotrimeric G_q and G_{11} ($G_{q/11}\alpha\beta\gamma$) to activate the expression of genes encoding the transcription factors *Dlx3*, *Dlx6*, *dHAND*, and *eHAND*. $Dlx6$ binds the pharyngeal arch enhancer (but not the cardiac enhancer) to stimulate transcription of *dHAND* (Charité et al., 2001). $G\alpha_q/G\alpha_{11}$ signaling may activate transcription of *Dlx3* and *Dlx6* and/or stabilize their mRNA. $G\alpha_q/G\alpha_{11}$ activates phospholipase $C\beta$ ($PLC\beta$)-catalyzed hydrolysis of phosphoinositide 4,5-bisphosphate (PIP_2) to produce the classical second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 binding to the IP_3 receptor (IP_3R) releases Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER) (reviewed in Muallem and Wilkie, 1999). These second messengers may activate target proteins that regulate *Dlx* and *HAND* gene expression.

pharyngeal arch expression of *dHAND* and is dependent on ET-1 signaling. *Dlx6* binds these sites in the *dHAND* pharyngeal arch enhancer and *Dlx6* expression is abolished in the pharyngeal arches of ET_A -null embryos (Charité et al., 2001). We have shown that *Dlx6* expression is $G\alpha_q/G\alpha_{11}$ -dependent, indicating that *dHAND* pharyngeal arch expression is controlled by *Dlx6* in response to $G\alpha_q/G\alpha_{11}$ -mediated ET-1 signaling. We propose that $G\alpha_q/G\alpha_{11}$ signaling activates the expression of *Dlx3* and *Dlx6*. Consequently, *Dlx6* binds the *dHAND* pharyngeal arch enhancer to drive expression of *dHAND* in the pharyngeal arches during craniofacial development (Fig. 5). Analysis of the *Dlx6* regulatory region may lead to identification of intermediate factor(s) directly affected by $G\alpha_q/G\alpha_{11}$ -mediated signaling.

Pharyngeal arch expression of *Msx1* has previously been reported to be dependent on *dHAND* and is absent in *dHAND*-null embryos (Thomas et al., 1998). Although neither *Dlx6* nor *dHAND* expression was detected in sections from $G\alpha_q^{(-/-)}G\alpha_{11}^{(-/-)}$ embryos, *Msx1* expression was still maintained. This is consistent with data from *endothelin-1*-null and ET_A -null embryos in which *Msx1* expression is normal, and low levels of *dHAND* expression are detectable (Clouthier et al., 1998; Thomas et al., 1998).

We have shown that ET-1-dependent genes require $G\alpha_q/G\alpha_{11}$ for correct expression in the pharyngeal arches. Ac-

tivation of these α -subunits is the first of many intracellular events occurring downstream of the endothelin receptor that ultimately lead to transcription of target genes such as *Dlx3*, *Dlx6*, and the *HAND* genes during craniofacial development.

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